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Patents ADP number *(if you know it)* 6997670005

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4. Title of the invention — Detection System

5. Name of your agent *(if you have one)* — ~~Carol P. Greaves~~ et al.

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DUPLICATE

Detection System

The present invention provides a method for detecting a target polynucleotide in a sample, for example by quantitatively monitoring an amplification reaction, as well as to probes and kits for use in these methods. The method is particularly suitable for the detection of polymorphisms or allelic variation and so may be used in diagnostic methods

Known fluorescence polymerase chain reaction (PCR) monitoring techniques include both strand specific and generic DNA intercalator techniques that can be used on a few second-generation PCR thermal cycling devices. These reactions are carried out homogeneously in a closed tube format on thermal cyclers. Reactions are monitored using a fluorimeter. The precise form of the assays varies but often relies on fluorescence energy transfer or FET between two fluorescent moieties within the system in order to generate a signal indicative of the presence of the product of amplification.

WO 99/28500 describes a very successful assay for detecting the presence of a target nucleic acid sequence in a sample. In this method, a DNA duplex binding agent and a probe specific for said target sequence, is added to the sample. The probe comprises a reactive molecule able to absorb fluorescence from or donate fluorescent energy to said DNA duplex binding agent. This mixture is then subjected to an amplification reaction in which target nucleic acid is amplified, and conditions are induced either during or after the amplification process in which the probe hybridises to the target sequence. Fluorescence from said sample is monitored.

As the probe hybridises to the target sequence, DNA duplex binding agent such as an intercalating dye is trapped between the strands. In general, this would increase the fluorescence at the wavelength associated with the dye. However, where the reactive molecule is able to absorb fluorescence from the dye

(i.e. it is an acceptor molecule), it accepts emission energy from the dye by means of FET, especially FRET, and so it emits fluorescence at its characteristic wavelength. Increase in fluorescence from the acceptor molecule, which is of a different wavelength to that of the dye, will indicate binding of the probe in duplex form.

Similarly, where the reactive molecule is able to donate fluorescence to the dye (i.e. it is a donor molecule), the emission from the donor molecule is reduced as a result of FRET and this reduction may be detected. Fluorescence of the dye is increased more than would be expected under these circumstances.

The signal from the reactive molecule on the probe is a strand specific signal, indicative of the presence of target within the sample. Thus the signal changes in fluorescence from the reactive molecule, which are indicative of the formation or destabilisation of duplexes involving the probe, are preferably monitored.

DNA duplex binding agents, which may be used in the process, are any entity which adheres or associates itself with DNA in duplex form and which is capable of acting as an energy donor or acceptor. Particular examples are intercalating dyes as are well known in the art.

The use of a DNA duplex binding agent such as an intercalating dye and a probe which is singly labelled is advantageous in that these components are much more economical than other assays in which doubly labelled probes are required. By using only one probe, the length of known sequence necessary to form the basis of the probe can be relatively short and therefore the method can be used, even in difficult diagnostic situations.

The DNA duplex binding agent used in the assay is typically an intercalating dye, for example SYBRGreen such as SYBRGreen I,

SYBRGold, ethidium bromide and YOPRO-1, which are themselves fluorescent.

In order for FET, such as FRET, to occur between the reactive molecule and the dye, the fluorescent emission of the donor (which may either be the intercalating dye or the reactive molecule on the probe) must be of a shorter wavelength than the acceptor (i.e. the other of the dye or the reactive molecule). The fluorescent signals produced by the molecules used as donor and/or acceptor can be represented as peaks within the visible spectrum.

Generally, there will be at least some overlap in the wavelengths of the emission. Even where the signals are sharp peaks, there will be some "leakage" of signal from fluorescent molecules so that it is generally necessary to resolve the strand specific peak produced by the probe from the DNA duplex binding agent signal. This can be done, for example by determining empirically the relationship between the spectra of the donor and acceptor and using this relationship to normalise the signals from the donor and acceptor.

The applicants have found an improved way of operating an assay of this type.

The present invention provides a method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising:

(a) adding to a sample suspected of containing said target nucleic acid sequence, a fluorescently labelled probe specific for said target sequence, and a DNA duplex binding agent which can absorb fluorescent energy from the fluorescent label on the probe but which does not emit visible light,

(b) subjecting the thus formed mixture to an amplification reaction in which target nucleic acid is amplified,

(c) subjecting said sample to conditions under which the said probe hybridises to the target sequence, and

(d) monitoring fluorescence from said sample.

The expression "visible light" used herein refers to radiation in the visible region of the spectrum, i.e. at wavelengths in the range of 390nm to 750nm.

By using a DNA duplex binding agent that does not emit light in the visible range of the spectrum, the problem with it supplying a signal that may overlap with that of the probe is avoided. Thus the need to resolve the signals from the probe from the signal from the DNA duplex binding agent is eliminated. This means that the apparatus, or at least the computational requirements placed upon the apparatus can be simplified.

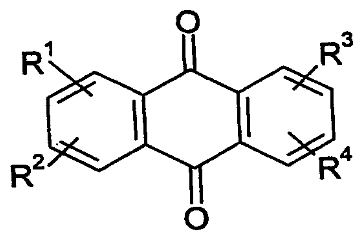
Alternatively, any areas of free bandwidth in the visible spectrum may be exploited by incorporating additional probes, which include different labels which fluoresce at different wavelengths so that more that one target may be monitored at the same time. This may be particularly useful in the case of multiplex PCR reactions.

The DNA duplex binding agent, which is used, may be an any compound which binds to a DNA duplex, provided it does not emit radiation in the visible portion of the spectrum. It may therefore be an intercalating agent, a minor groove binder, a compound which binds to DNA major groove, or a compound which binds or stacks onto an end base of a probe, as well as combinations therof. In particular embodiments, it will comprise an intercalating agent or a minor groove binder. It may emit radiation at wavelengths outside the visible range of the spectrum, for example in the infrared range. However, such emissions would not be detectable in the context of the method of the invention, and so effectively the DNA duplex binding agent acts only as a "dark quencher".

Examples of suitable DNA binding agents, which may be used in this way, include DNA binding agents that have conjugated

aromatic ring systems. Rings may be aryl rings, such as phenyl, napthyl or anthracene rings, or aromatic heterocyclic rings, for example containing up to 20 atoms, up to five of which are heteroatoms such as oxygen, sulphur and nitrogen. Examples of such systems include anthracyclins or anthraquinones. These may be substituted to provide the appropriate DNA binding properties.

In particular, compounds comprise an optionally substituted anthraquinone of structure (I)



(I)

where R^1, R^2, R^3 and R^4 are independently selected from hydrogen, a functional group, or a hydrocarbyl group optionally substituted by for example functional groups, or R^1 and R^2 or R^3 and R^4 are optionally joined together to form a ring which optionally contains heteroatoms, and/or is optionally substituted by a functional group or a hydrocarbyl group.

As used herein, the term "functional group" refers to a reactive group, which suitably contains a heteroatom. Examples of functional groups include halo, cyano, nitro, oxo, $-OC(O)R^a$, $-OR^a$, $-C(O)OR^a$, $S(O)_tR^a$, NR^bR^c, $OC(O)NR^bR^c$, $C(O)NR^bR^c$, $OC(O)NR^bR^c$, $-NR^7C(O)_{n'}R^6$, $-NR^aCONR^bR^c$, $-C{=}NOR^a$, $-N{=}CR^bR^c$, $S(O)_tNR^bR^c$, $C(S)_nR^a$, $C(S)OR^a$, $C(S)NR^bR^c$ or $-NR^bS(O)_tR^a$ where R^a , R^b and R^c are independently selected from hydrogen or optionally substituted hydrocarbyl, or R^b and R^c together form an optionally substituted ring which optionally contains further heteroatoms such as $S(O)_s$, oxygen and nitrogen, n' is an integer of 1 or 2, s is 0, 1 or 2, t is 0 or an integer of 1-3.

Suitable optional substituents for hydrocarbyl groups R^a, R^b and R^c may also be functional groups.

As used herein the term "hydrocarbyl" refers to organic groups comprising carbon and hydrogen atoms such as alkyl, alkenyl, alkynyl, cycloalkyl, aryl or aralkyl. The term "alkyl" refers to straight or branched chain alkyl group, suitably containing up to 20, more suitably up to 10 and preferably up to 6 carbon atoms. The term "alkenyl" or "alkynyl" refers to unsaturated straight or branched chains, having from 2 to 10 carbon atoms. The term "cycloalkyl" refers to alkyl groups which have at least 3 carbon atoms, and which are cyclic in structure. The term "aryl" refers to aromatic rings such as phenyl and naphthyl. The term aralkyl refers to alkyl groups substituted by aryl groups such as benzyl.

Particular examples of substituents for R^1, R^2, R^3 and R^4 are hydroxy groups so as to give rise to keto-enol tautomerism.

Preferably the compound contains one or more heteroatoms, to give a charge which will assist in binding to DNA. The heteroatoms, such as oxygen, nitrogen or sulphur, may be included in the substituent side chains. In particular embodiments, the compounds of formula (I) include at least one nitrogen atom within the substituents R^1, R^2, R^3 and R^4.

Examples of such compounds may be found in the pharmaceutical fields, and in particular in anticancer or antibiotic applications, as a result of the DNA binding functionality. For examples, compounds which may have the properties which make them suitable for use as DNA binding agents in the assay of the present invention include US Patent No. 4197249, US Patent No 3183157, US Patent No 4012284 and US Patent No.3997662

Particular examples are mitoxantrone (1,4-dihydroxy 5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) or it salt such as the hydrochloride or dihydrochloride salt, nogalamycin (2R-(2α,3β,4α,5β,6α,11β,13α,14α)]-11-[6-deoxy-3-C-mehtyl-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-4-

(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2H-naphthaceno[1,2-b]oxocin-14-carboxylic acid methyl ester) or daunomycin (8S,-cis)-8-acetyl-10-[3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacendione).

In a particular embodiment, the DNA duplex binding agent is mitoxantrone.

Alternatively or additionally a quenching moiety such as 4-(4-dimethylaminophenylazo) benzoic acid (DABCYL) may be attached and preferably covalently bound, to a known DNA binding, intercalating or minor or major groove binding agent. In this case, the DNA binding agent may have some degree of fluorescence provided that this is entirely quenched by the quenching moiety.

These compounds have the effect of stabilising the duplex. This is advantageous in two respects. Firstly it improves the binding of the probe to the target, reducing the time taken to change temperatures during the amplification, and so allowing the reaction to be carried out faster. Secondly it allows the use of shorter nucleic acid sequences for primers and probes. This is generally useful where for example melt point analysis is being carried out, since the shorter the probe, the more significant will be the difference between melting points caused by mismatches. It may be particularly useful in for example AT rich targets where long primers and probes can reduce the specificity of the reaction because of the low temperatures that may be required for probe and primer annealing.

The quenching effects of the DNA duplex binding agent may be felt to some extent by the probe when in single stranded form. However, the quenching will be significantly and distinguishably more pronounced in the case of duplex DNA. Generally any free label present in the system will not be subject to quenching by

the DNA duplex binding agent, since no association forms between them.

The amount of DNA duplex binding agent which is added to the reaction mixture is suitably sufficient to cause measurable quenching of the signal from the fluorescent label, but not sufficient to inhibit amplification. The range of concentrations which will achieve this vary depending upon the precise DNA duplex binding agent used, and can be determined by routine methods as illustrated hereinafter. For DNA duplex binding agents such as mitoxantrone or daunomycin, concentrations of the order of 1μM to 100μM and suitably about 10μM-25μM would be employed.

The method of the invention is extremely versatile in its applications. The method can be used to generate both quantitative and qualitative data regarding the target nucleic acid sequence in the sample, as discussed in more detail hereinafter. In particular, not only does the invention provide for quantitative amplification, but also it can be used, additionally or alternatively, to obtain characterising data such as duplex destabilisation temperatures or melting points.

In the method of the invention, the sample may be subjected to conditions under which the probe hybridises to the samples before, during or after the amplification reaction. The process therefore allows the detection to be effected in a homogenous manner, in that the amplification and monitoring can be carried out in a single container with all reagents added initially. No subsequent reagent addition steps are required. Neither is there any need to effect the method in the presence of solid supports (although this is an option).

The probe may comprise a nucleic acid molecule such as DNA or RNA, which will hybridise to the target nucleic acid sequence when the latter is in single stranded form. In this instance,

step (c) will involve the use of conditions which render the target nucleic acid single stranded.

Probe may either be free in solution or immobilised on a solid support, for example to the surface of a bead such as a magnetic bead, useful in separating products, or the surface of a detector device, such as the waveguide of a surface plasmon resonance detector. The selection will depend upon the nature of the particular assay being looked at and the particular detection means being employed.

In particular, the amplification reaction used will involve a step of subjecting the sample to conditions under which any of the target nucleic acid sequence present in the sample becomes single stranded. Such amplification reactions include the polymerase chain reaction (PCR) or the ligase chain reaction (LCR), but is preferably a PCR reaction.

It is possible then for the probe to hybridise during the course of the amplification reaction provided appropriate hybridisation conditions are encountered.

In a preferred embodiment, the probe may be designed such that these conditions are met during each cycle of the amplification reaction. Thus at some point during each cycle of the amplification reaction, the probe will hybridise to the target sequence, and whereupon the fluorescent signal will be quenched as a result of its close proximity to the DNA duplex binding agent trapped between the probe and the target sequence. As the amplification proceeds, the probe will be separated or melted from the target sequence and so the signal generated by it will be restored. Hence in each cycle of the amplification, a fluorescence peak from the fluorescent label at the point at which the probe is annealed is generated. The intensity of the peak will decrease as the amplification proceeds because more target sequence becomes available for binding to the probe.

By monitoring the fluorescence of the fluorescent label in the sample during each cycle, the progress of the amplification reaction can be monitored in various ways. For example, the data provided by melting peaks can be analysed, for example by calculating the area under the melting peaks and this data plotted against the number of cycles.

Fluorescence is suitably monitored using a known fluorimeter. The signals from these, for instance in the form of photo-multiplier current, are sent to a data processor board and converted into a spectrum associated with each sample tube. Multiple tubes, for example 96 tubes, can be assessed at the same time. Data may be collected in this way at frequent intervals, for example once every 10ms, throughout the reaction.

This data provides the opportunity to quantitate the amount of target nucleic acid present in the sample.

In addition, the kinetics of probe hybridisation will allow the determination, in absolute terms, of the target sequence concentration. Changes in fluorescence from the sample can allow the rate of hybridisation of the probe to the sample to be calculated. An increase in the rate of hybridisation will relate to the amount of target sequence present in the sample. As the concentration of the target sequence increases as the amplification reaction proceeds, hybridisation of the probe will occur more rapidly. Thus this parameter also can be used as a basis for quantification. This mode of data processing useful in that it is not reliant on signal intensity to provide the information.

Suitable fluorescent labels are rhodamine dyes or other dyes such as Cy5, fluorescein or derivatives thereof. These may be attached to the probe in a conventional manner. The position of the fluorescent label along the probe is immaterial although it general, they will be positioned at an end region of the probe.

Preferably they are positioned at the 3′ end of the probe, as they will then act as a steric blocking agent, to prevent extension of the probe by the polymerase during the amplification. This may avoid the need to take other measures, such as phosphorylation, in order to block the 3′ end of the probe.

It is possible to design the probe and the assay conditions such that the probe is hydrolysed by the DNA polymerase used in the amplification reaction, thereby releasing the fluorescent label. In this case, the probe will be designed to bind during the annealing and extension phase of the PCR reaction and the polymerase used in the assay will be one which has 5′-3′exonuclease activity. The released fluorescent label produces an increasing signal since it is no longer quenched by the DNA duplex binding agent. In this case therefore, the reaction can be monitored by observing the increasing signal of the free fluorescent label. The signal must be monitored at temperatures that are above those where the probe interacts with the target or product.

However, it is not necessary in this assay for the probe to be consumed in this way as signal production can be achieved without dissociating the probe.

In order to achieve a fully reversible signal which is directly related to the amount of amplification product present at each stage of the reaction, and/or where speed of reaction is of the greatest importance, for example in rapid PCR, it is preferable that the probe is designed such that it is released intact from the target sequence. This may be, for example, during the extension phase of the amplification reaction. However, since the signal is not dependent upon probe hydrolysis, the probe may be designed to hybridise and melt from the target sequence at any stage during the amplification cycle. For example probes which hybridise most strongly at a stage other than the

extension phase of the cycle will ensure that interference with the amplification reaction is minimised.

Where probes which bind strongly at or below the extension temperature are used, their release intact from the target sequence can be achieved by using a 5′-3′ exonuclease lacking enzyme such as Stoffle fragment of Taq or Pwo, as the polymerase in the amplification reaction.

The probe may then take part again in the reaction, and so represents an economical application of probe.

The data generated in this way using probes which reversibly hybridise to the target and are not hydrolysed, can be interpreted in various ways. In its simplest form, a decrease in fluorescence of the fluorescent label at the probe annealing temperature in the course of or at the end of the amplification reaction is indicative of an increase in the amount of the target sequence present, suggestive of the fact that the amplification reaction has proceeded and therefore the target sequence was in fact present in the sample.

However, as outlined above, quantification is also possible by monitoring the amplification reaction throughout.

Finally, it is possible to obtain characterisation data and in particular melting point analysis, either as an end point measure or throughout, in order to obtain information about the sequence as will be discussed further below.

Thus, a preferred embodiment of the invention comprises a method for detecting nucleic acid amplification comprising: performing nucleic acid amplification on a target polynucleotide in the presence of (a) a nucleic acid polymerase (b) at least one primer capable of hybridising to said target polynucleotide, (c) an oligonucleotide probe which is capable of binding to said target polynucleotide sequence and which contains a fluorescent

label and (d) a DNA duplex binding agent which is capable of absorbing fluorescent energy from the said fluorescent label, and which does not emit light in the visible range of the spectrum; and monitoring changes in fluorescence during the amplification reaction.

The amplification is suitably carried out using a pair of primers which are designed such that only the target nucleotide sequence within a DNA strand is amplified as is well understood in the art. The nucleic acid polymerase is suitably a thermostable polymerase such as Taq polymerase.

Suitable conditions under which the amplification reaction can be carried out are well known in the art. The optimum conditions may be variable in each case depending upon the particular amplicon involved, the nature of the primers used and the enzymes employed. The optimum conditions may be determined in each case by the skilled person. Typical denaturation temperatures are of the order of 95°C, typical annealing temperatures are of the order of 55°C and extension temperatures are of the order of 72°C.

The method can be used in hybridisation assays for determining characteristics of particular sequences.

Thus in a further aspect, the invention provides a method for determining a characteristic of a sequence, said method comprising;
a) adding to a sample suspected of containing said sequence, a fluorescently labelled probe specific for said target sequence and a DNA duplex binding agent able to absorb fluorescence from a fluorescent label on the probe but which does not emit radiation in the visible range of the spectrum,
(b) subjecting said sample to conditions under which the said probe hybridises to the target sequence,
(c) monitoring fluorescence from said sample and determining a particular reaction condition, characteristic of said sequence,

at which fluorescence changes as a result of the hybridisation of the probe to the sample or destabilisation of the duplex formed between the probe and the target nucleic acid sequence.

Suitable reaction conditions include temperature, electrochemical, or the response to the presence of particular enzymes or chemicals. By monitoring changes in fluorescence as these properties are varied, information characteristic of the precise nature of the sequence can be determined. For example, in the case of temperature, the temperature at which the probe separates or "melts" from the target sequence can be determined. This can be extremely useful in for example, to detect and if desired also to quantitate, polymorphisms in sequences including allelic variation in genetic diagnosis. By "polymorphism" is included transitions, transversions, insertions, deletions or inversions which may occur in sequences, particularly in nature.

The hysteresis of melting of the probe will be different if the target sequence varies by only one base pair. Thus where a sample contains only a single allelic variant, the temperature of melting of the probe will be a particular value which will be different from that found in a sample which contains only another allelic variant. A sample containing both allelic variants which show two melting points corresponding to each of the allelic variants.

Similar considerations apply with respect to electrochemical properties, or in the presence of certain enzymes or chemicals. The probe may be immobilised on a solid surface across which an electrochemical potential may be applied. Target sequence will bind to or be repulsed from the probe at particular electrochemical values depending upon the precise nature of the sequence.

This embodiment can be effected in conjunction with amplification reactions such as the PCR reaction mentioned above, or it may be employed individually.

Further aspects of the invention include kits for use in the method of the invention. These kits will contain a DNA duplex binding agent which able to absorb fluorescent energy from a fluorescent label which may be found on a probe, but which does not emit light in the visible range of the spectrum. Other potential components of the kit include reagents used in amplification reactions such as DNA polymerase (including chemically modified TAQ for "hotstart" reactions), primers, buffers and adjuncts known to improve the PCR process such as the "hotstart" reagents such as antiTaq antibody, or pyrophosphate and a pyrophosphatase, as described in copending International Patent Application PCT/GB02/01861. The kit may additionally or alternatively include a probe for a target sequence which is fluorescently labelled.

The kits may include all the reagents together in a single container, or some may be in separate containers for mixing on site.

In a further aspect, the invention provides the use of a DNA duplex binding agent which can absorb fluorescent energy but which does not emit visible light in a method for detecting the presence of a target nucleic acid sequence in a sample. Suitable methods are as defined above. Particular examples of DNA duplex binding agents are also described above.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows diagrammatically the interactions which occur using the method of the invention;

Figure 2 illustrates stages during an amplification reaction in accordance with the invention;

Figures 3 is a graph showing the results of an amplification reaction in accordance with the invention, plotting the inverse of fluorescence occurring at the end of the annealing step, against cycle number, and illustrating the effect of 1:100 of 0.0193M mitoxantrone on three 10 fold dilutions of human placental DNA;

Figure 4 is a graph showing the quenching effect of a 10 fold dilution series of the neat (0.0193M) mitoxantrone on the CTW19 probe; and

Figure 5 is a graph showing the quenching effect of a 10 fold dilution series of the neat (5mM) daunomcyin on the CTW19 probe.

An element of the method of the invention is a probe (1) which carries a fluorescent label (2), preferably at the 3' end. This probe, which specifically binds the target sequence, is added to the sample suspected of containing the target sequence together with a DNA duplex binding agent (3).

When the probe (1) is free in solution, the fluorescent label (2) will fluoresce. Some DNA duplex binding agent may become associated with the probe which may quench the signal slightly, but the level of quenching is low (Figure 1A). However, when the probe (1) hybridises with a single stranded target sequence (4) to form a duplex as illustrated in Figure 1B, DNA duplex binding agent (3) becomes associated with the duplex and is therefore brought into close proximity to the fluorescent label. Fluorescent energy from the label passes to the DNA duplex binding agent (3), and so the fluorescence from the sample is reduced or quenched. Decrease in the fluorescence of the label will thus be indicative of hybridisation of the probe to the target sequence.

Thus by measuring the decrease in fluorescence of the label, for example as the temperature decreases, the point at which hybridisation occur can be detected. Similarly, an increase in

label fluorescence will occur as the temperature increases at the temperature at which the probe (1) melts from the target sequence (4), as the label is no longer affected by the DNA duplex binding agent.

The melt temperature will vary depending upon the hybridisation characteristics of the probe and the target sequence. For example, a probe, which is completely complementary to a target sequence, will melt at a different temperature to a probe that hybridises with the target sequence but contains one or more mismatches.

Figure 2 illustrates how the method of the invention can be employed in amplification reactions such as the PCR reaction. Probe (1) will hybridise to single stranded DNA in conjunction with the DNA duplex binding agent (3) and thus the label signal will be quenched (Figure 2A). In the illustrated embodiment this occurs during the annealing phase of the cycle during which the primer (5) anneals. As the amount of target sequence increases as a result of the amplification, the signal generated during the annealing phase by the label will decrease as a result of increased quenching by the formation of more duplexes which incorporate the probe and also the DNA duplex binding agent.

During the extension phase, the probe is removed from the target sequence because the DNA polymerase displaces it. At this point, the label signal increases because the probe moves away from the DNA duplex binding agent (Figure 2B).

By monitoring the fluorescence from the label, the progress of the amplification reaction can be followed and the quantity of target sequence present in the original sample can be determined.

Example 1

PCR amplification reaction

The method of the invention was tested using the Carl Wittwer assay for the human beta Globin gene. In each case, the following experimental protocol was followed.

First of all, 10mls of a 2x Master mix formulation was prepared comprising the following components:

2x Master Mix Formulation: 2000µl Tris pH 8.8 at 500mM
2000µl dUTP Nucleotides at 2mM
250µl B.S.A at 20mg/ml
1600µl Glycerol
200µl Uracil-N-Glycosylase at 1 unit/µl
160µl Taq Polymerase at 5 units/µl
3190µl HPLC Grade Water
600µl Magnesium Chloride solution at 0.1M

A PCR mix formulation, suitable for conducting the Carl Wittwer assay, was then prepared and comprised the following components:

PCR Mix Formulation: 50µl of 2x Master mix at 3mM Mg^{2+}
10µl of Forward Primer (PCO3) at 10µM
10µl of Reverse Primer (PCO4) at 10µM
10µl of Probe (CTW19) at 2µM
5µl of HPLC Grade Water
5µl of Mitoxantrone at 10µM concs

Primer sequence (PCO3): ACA CAA CTG TGT TCA CTA GC
Primer sequence (PCO4): CAA CTT CAT CCA CGT TCA CC
CTW19: CAA ACA GAC ACC ATG GTG CAC CTG ACT CCT GAG GAT (3' fluorescein)

This PCR mix formulation constituted 90µl in total. The mix was then vortexed thoroughly and split into 2 x 45µl. To one of these was added 5µl of HPLC grade water to act as No Template

Control's (NTC's) and to the other 5µl of human placental DNA (Various Concentrations) was added to act as the Positives. These 2 x 50µl were then further split into 4 x 20µl and pipetted into Lightcycler capillaries to create NTC's in duplicate and +'s in duplicate

The above mix would be made for each value of the variable(s) being tested in each experiment.

The capillaries were then spun down and run on the Roche Lightcycler on the following cycle programme:

<u>Carry over prevention x 1</u>
50°C for 60 seconds
95°C for 15 seconds
<u>Cycle x 50</u>
95°C for 5 seconds
60°C for 5 seconds. Fluorescence collected at this step in F1 channel (530nm)
74°C for 5 seconds
<u>Melt analysis x 1</u>
50°C for 15 seconds
Slow ramp to 95°C at 0.1°C/second. Fluorescence collected throughout this step in F1 channel (530nm)

A typical result is shown in Figure 3.

Figure 3 illustrates that for a 10 fold dilution series, a distinguishable signal, above that of background. A tenfold dilution of target template in an optimium PCR, where the amplification would be such that exponential amplification occurs, would result in increase in the number of amplicons by a factor of 2 every cycle. A probe system that is used to detect the concentration of amplicons, and by inference the initial amount of target, should generate signals that will rise above background at an arbitary cycle values that are ~3.31 cycles

apart for each 10 fold dilution within the functional range of the PCR. This is clearly shown in Figure 3.

Example 2

Determination of Optimum concentration of DNA duplex binding agents

The PCR reaction as described in Example 1 was repeated using various concentrations of DNA duplex binding agents, mitoxantrone and daunomcyin. The results are shown in Figures 4 and 5 respectively. It is clear from these Figures that clear signals representing the amplification reaction appeared where the starting mitoxantrone material (0.0193M) had been effectively diluted by 1:100 before being added to the reaction mixture in a 1 in 20 dilution, resulting in a final concentration of about 10μM.

Similarly the 5mM daunomycin starting material was diluted by 1:10 before further dilution (1:20) in the PCR reaction mixture. The final concentration in this case was 25μM.

Claims

1. A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising:
(a) adding to a sample suspected of containing said target nucleic acid sequence, a fluorescently labelled probe specific for said target sequence, and a DNA duplex binding agent which can absorb fluorescent energy from the fluorescent label on the probe but which does not emit visible light,
(b) subjecting the thus formed mixture to an amplification reaction in which target nucleic acid is amplified,
(c) subjecting said sample to conditions under which the said probe hybridises to the target sequence, and
(d) monitoring fluorescence from said sample.

2. A method according to claim 1 wherein the DNA duplex binding agents has a fused conjugated ring system.

3. A method according to claim 1 or claim 2 wherein the DNA duplex binding agent is mitoxantrone (1,4-dihydroxy 5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) or it salt such as the hydrochloride or dihydrochloride salt, nogalamycin (2R-(2α,3β,4α,5β,6α,11β,13α,14α)]-11-[6-deoxy-3-C-mehtyl-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-4-(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2H-naphthaceno[1,2-b]oxocin-14-carboxylic acid methyl ester) or daunomycin (8S,-cis)-8-acetyl-10-[3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacendione).

4. A method according to claim 3 wherein the DNA binding agent is mitoxantrone.

5. A method according to any one of the preceding claims wherein the target nucleic acid is rendered single stranded prior to hybridisation to the probe in step (c).

6. A method according to any one of the preceding claims wherein the amplification reaction is the polymerase chain reaction (PCR).

7. A method according to any one of the preceding claims wherein the probe hybridises with the target nucleic acid during every cycle of the amplification reaction.

8. A method according to claim 7 wherein the fluorescence from the sample is monitored throughout the amplification reaction.

9. A method according to claim 8 wherein fluorescence data generated is used to determine the rates of probe hybridisation.

10. A method according to any one of claims 7 to 9 wherein the fluorescence data is used to quantitate the amount of target nucleic acid present in the sample.

11. A method according to any one of the preceding claims wherein the fluorescent label is a rhodamine dye, Cy5, fluorescein or a fluorescein derivative.

12. A method according to any one of the preceding claims wherein the fluorescent label is attached at an end region of the probe.

13. A method according to claim 13 wherein the fluorescent label is attached at the 3′end of the probe and prevents extension thereof by a polymerase.

14. A method according to anyone of the preceding claims wherein the probe is designed such that it is released intact from the target sequence during a phase of the amplification process other than the extension phase.

15. A method according to any one of claims 1 to 13 wherein the probe is designed such that it is released intact from the

target sequence during the extension phase of the amplification process by the action of the polymerase, and the amplification reaction is effected using a polymerase which lacks 5'-3' exonuclease activity.

16. A method according to claim 1 which comprises performing nucleic acid amplification on a target polynucleotide in the presence of (a) a nucleic acid polymerase (b) at least one primer capable of hybridising to said target polynucleotide, (c) an oligonucleotide probe which is capable of binding to said target polynucleotide sequence and which contains a fluorescent label and (d) a DNA duplex binding agent which is capable of absorbing fluorescent energy from the said fluorescent label, and which does not emit light in the visible range of the spectrum; and monitoring changes in fluorescence during the amplification reaction.

17. A method according to claim 16 wherein the amplification is suitably carried out using a pair of amplification primers.

18. A method according to claim 16 or claim 17 wherein the nucleic acid polymerase is a thermostable polymerase.

19. A method according to anyone of the preceding claims wherein in a further step, a hybridisation assay is carried out and a hybridisation condition which is characteristic of the sequence is measured.

20. A method according to claim 19 wherein the condition is temperature, electrochemical potential, or reaction with an enzyme or chemical.

21. A method according to claim 20 wherein the condition is temperature.

22. A method according to claim 21 which is used to detect allelic variation or a polymorphism in a target sequence.

23. A method for determining a characteristic of a sequence, said method comprising;
a) adding to a sample suspected of containing said sequence, a fluorescently labelled probe specific for said target sequence and a DNA duplex binding agent able to absorb fluorescence from a fluorescent label on the probe but which does not emit radiation in the visible range of the spectrum,
(b) subjecting said sample to conditions under which the said probe hybridises to the target sequence,
(c) monitoring fluorescence from said sample and determining a particular reaction condition, characteristic of said sequence, at which fluorescence changes as a result of the hybridisation of the probe to the sample or destabilisation of the duplex formed between the probe and the target nucleic acid sequence.

24. A method according to claim 23 wherein the reaction condition characteristic of said sequence is temperature, electrochemical potential, or reaction with an enzyme or chemical.

25. A method according to claim 24 wherein the condition is temperature.

26. A method according to any one of claims 23 to 25 wherein the results obtained from two sequences are compared in order to determine the presence of polymorphisms or variations therebetween.

27. A method according to any one of claims 23 to 26 wherein the DNA duplex binding agent is mitoxantrone (1,4-dihydroxy 5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) or it salt such as the hydrochloride or dihydrochloride salt, nogalamycin (2R-(2α,3β,4α,5β,6α,11β,13α,14α)]-11-[6-deoxy-3-C-mehtyl-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-4-(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2H-

naphthaceno[1,2-b]oxocin-14-carboxylic acid methyl ester) or daunomycin (8S,-cis)-8-acetyl-10-[3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacendione).

28. A kit for use in the method according to any one of the preceding claims, which kit comprises (i) a DNA duplex binding agent which is able to absorb fluorescent energy but which does not emit radiation in the visible range of the spectrum, and either (ii)a fluorescently labelled probe specific for a target nucleotide sequence, or (iii) one or more reagents necessary for conducting an amplification reaction.

29. A kit according to claim 28 which contains (iii) and wherein the reagents are selected from primers, DNA polymerase, buffers, or adjuncts known to improve PCR.

30. A kit according to claim 28 or claim 29 wherein the DNA duplex binding agent is mitoxantrone (1,4-dihydroxy 5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) or it salt such as the hydrochloride or dihydrochloride salt, nogalamycin (2R-(2α,3β,4α,5β,6α,11β,13α,14α)]-11-[6-deoxy-3-C-mehtyl-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-4-(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2H-naphthaceno[1,2-b]oxocin-14-carboxylic acid methyl ester) or daunomycin (8S,-cis)-8-acetyl-10-[3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacendione).

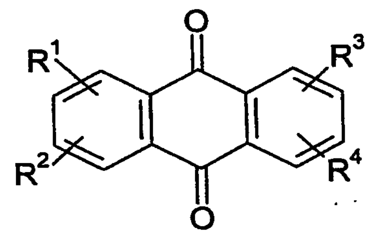
31. A kit according to any one of claims 28 to 30 which comprises both (i) and (ii).

32. The use of a DNA duplex binding agent which can absorb fluorescent energy but which does not emit visible light in a method for detecting the presence of a target nucleic acid sequence in a sample.

33. The use according to claim 32 wherein the DNA duplex binding agent comprises a conjugated aromatic ring system.

34. The use according to claim 33 wherein the DNA duplex binding agent comprises an anthracyclin or anthraquinone.

35. The use according to any one or claims 32 to 34 wherein the DNA duplex binding agent is an optionally substituted anthraquinone of structure (I)



(I)

where R^1, R^2, R^3 and R^4 are independently selected from hydrogen, a functional group, or a hydrocarbyl group optionally substituted by for example functional groups, or R^1 and R^2 or R^3 and R^4 are optionally joined together to form a ring which optionally contains heteroatoms, and/or is optionally substituted by a functional group or a hydrocarbyl group.

36. The use according to any one of claims 32 to 35 wherein the DNA duplex binding agent is mitoxantrone (1,4-dihydroxy 5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) or it salt such as the hydrochloride or dihydrochloride salt, nogalamycin (2R-(2α,3β,4α,5β,6α,11β,13α,14α)]-11-[6-deoxy-3-C-mehtyl-2,3,4-tri-O-methyl-α-L-mannopyranosyl)oxy]-4-(dimethylamino)-3,4,5,6,9,11,12,13,14,16-decahydro-3,5,8,10,13-pentahydroxy-6,13-dimethyl-9,16-dioxo-2,6-epoxy-2H-naphthaceno[1,2-b]oxocin-14-carboxylic acid methyl ester) or daunomycin (8S,-cis)-8-acetyl-10-[3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12-naphthacendione).

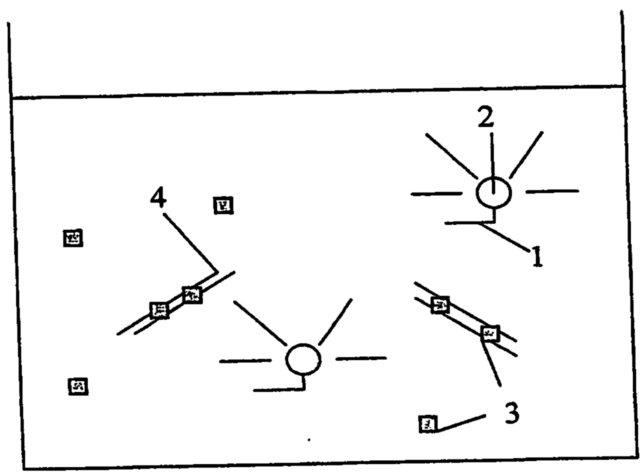
37. The use according to claim 36 wherein the DNA duplex binding agent is mitoxantrone.

Abstract

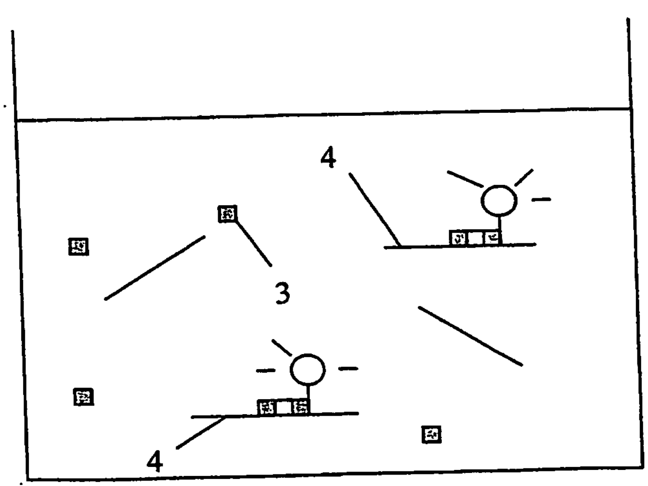
A method for detecting the presence of a target nucleic acid sequence in a sample, said method comprising:
(a) adding to a sample suspected of containing said target nucleic acid sequence, a fluorescently labelled probe specific for said target sequence, and a DNA duplex binding agent which can absorb fluorescent energy from the fluorescent label on the probe but which does not emit visible light,
(b) subjecting the thus formed mixture to an amplification reaction in which target nucleic acid is amplified,
(c) subjecting said sample to conditions under which the said probe hybridises to the target sequence, and
(d) monitoring fluorescence from said sample.

This method can be used for example to monitor amplification reactions such as PCR reactions, such that the amount of target sequence present in the sample may be determined. Additionally or alternatively, it may be used to generate duplex destabilisation data such as melt hysteresis information for amplification monitoring or for detection and quantification of polymorphisms or allelic variation, and so is useful in genetic diagnosis.

Figure 1



A



B

Figure 2A

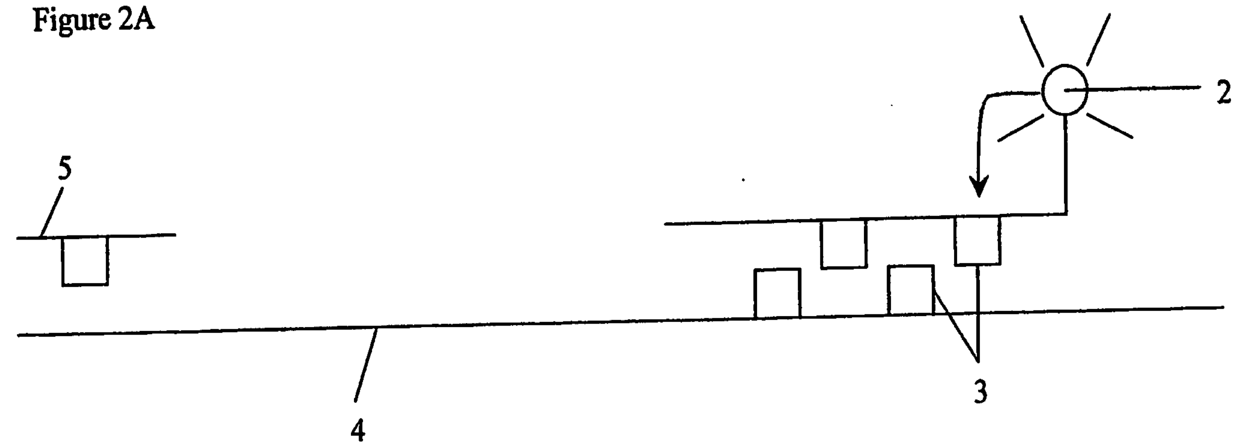


Figure 2B

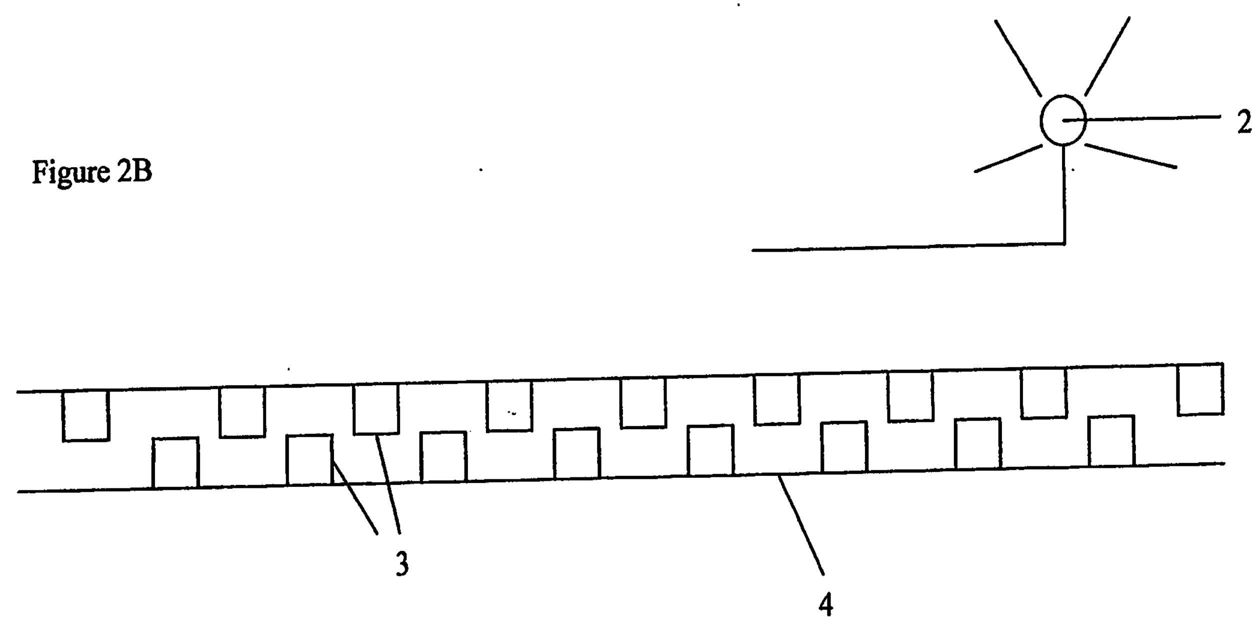
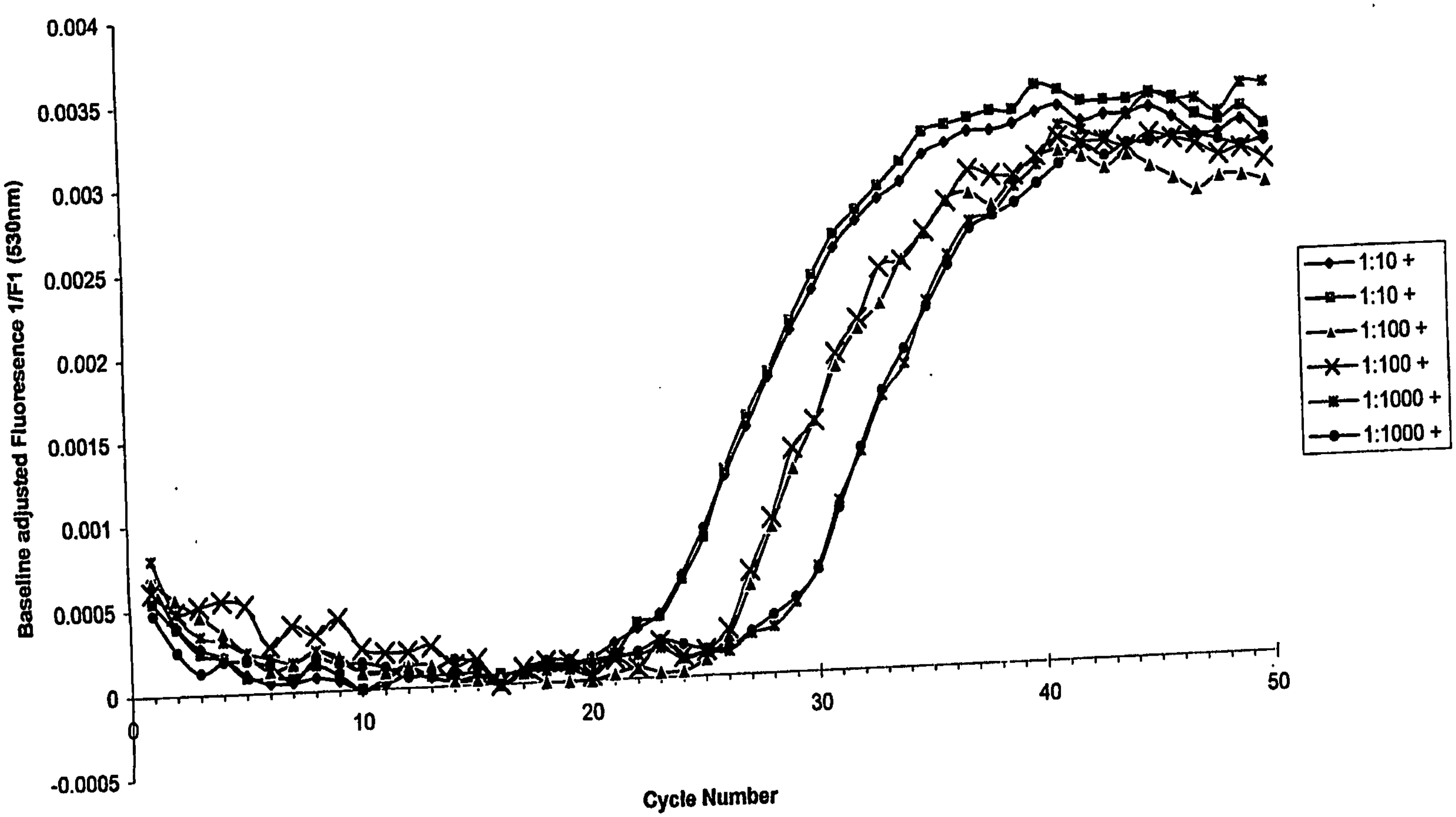


Figure 3



0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
-0.0005
Baseline adjusted Fluoresence 1/F1 (530nm)
0
10
20
30
40
50
Cycle Number
1:10 +
1:10 +
1:100 +
1:100 +
1:1000 +
1:1000 +

Figure 4

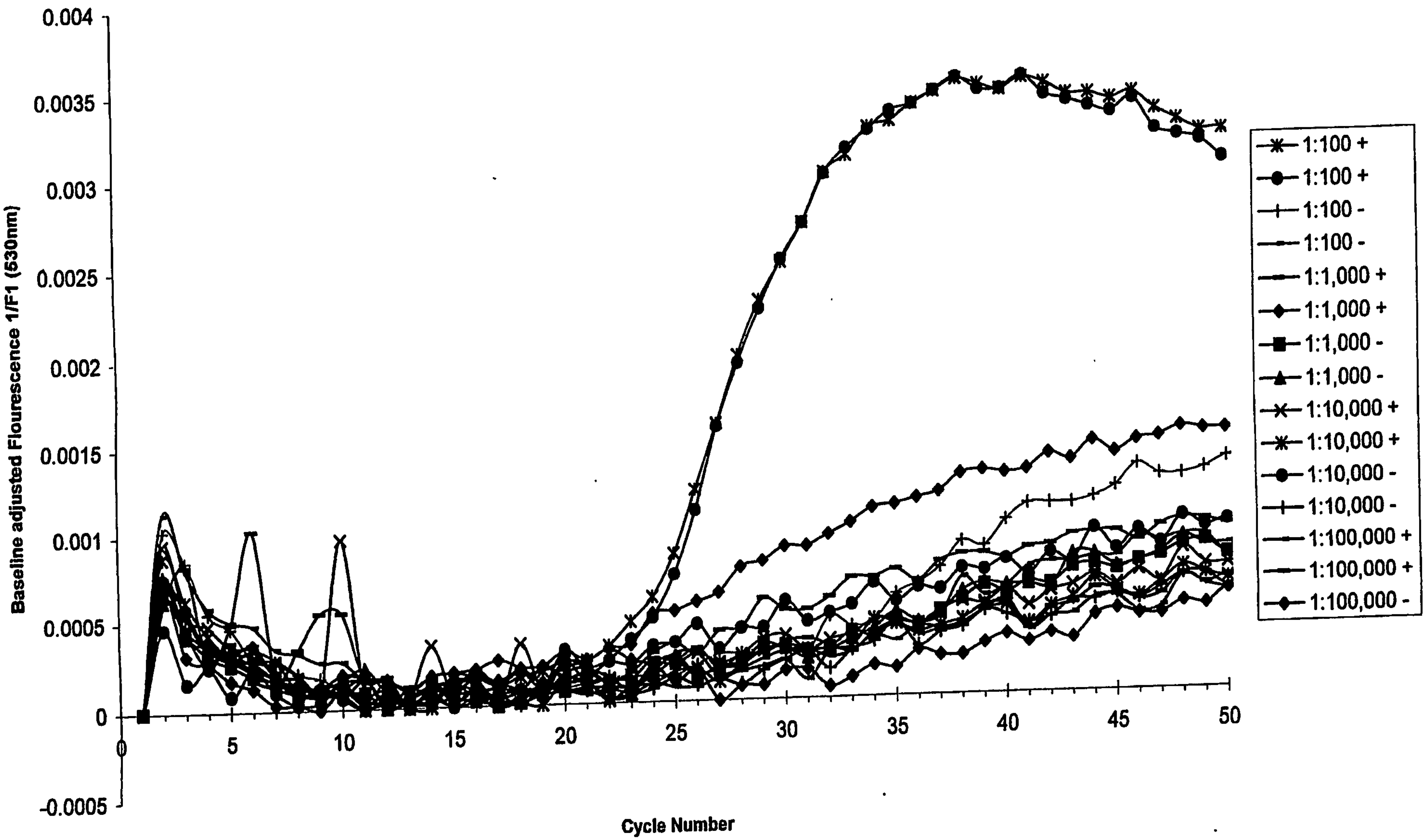
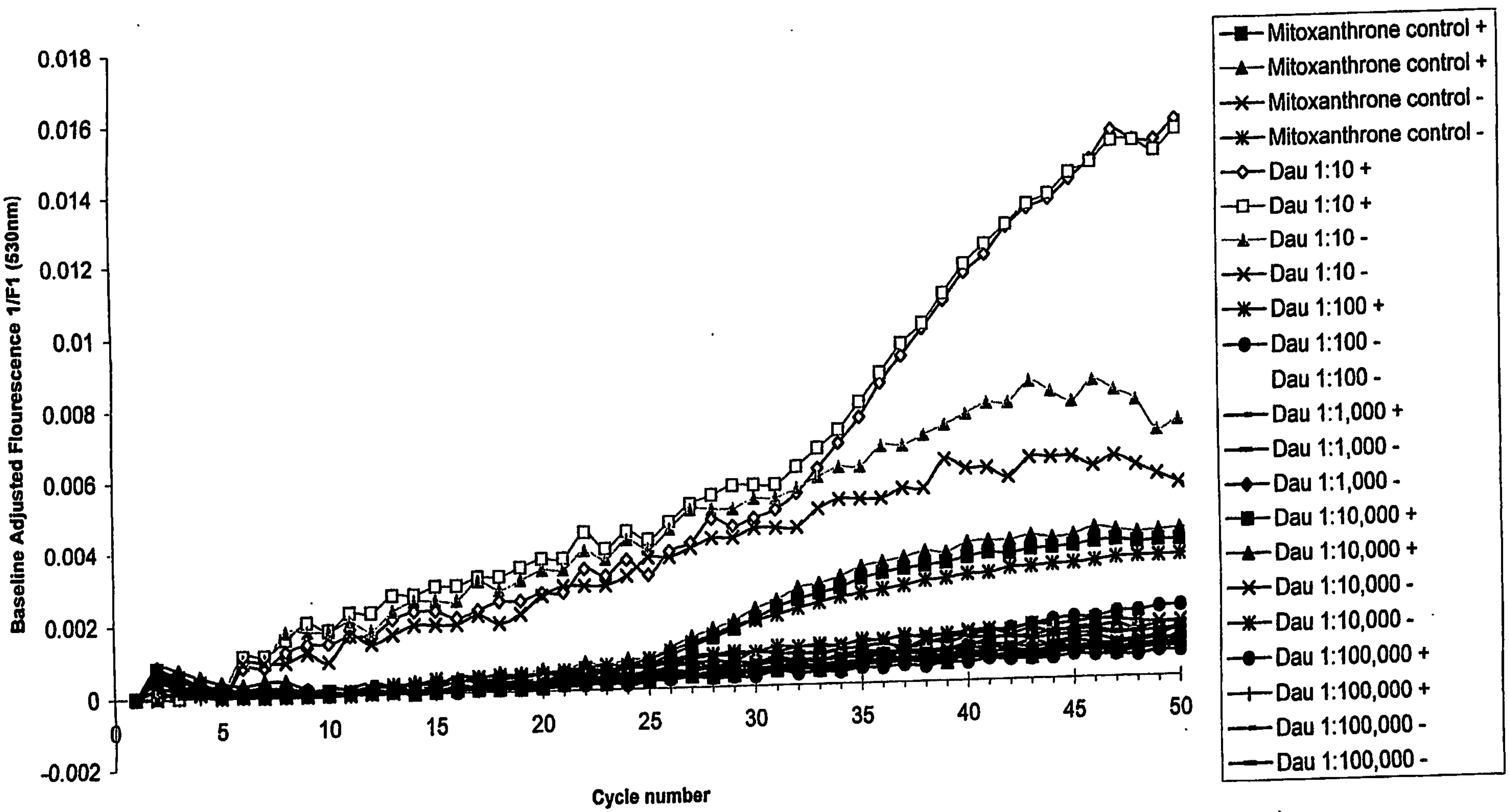

Baseline adjusted Flourescence 1/F1 (530nm)
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
-0.0005
0
5
10
15
20
25
30
35
40
45
50
Cycle Number
1:100 +
1:100 +
1:100 -
1:100 -
1:1,000 +
1:1,000 +
1:1,000 -
1:1,000 -
1:10,000 +
1:10,000 +
1:10,000 -
1:10,000 -
1:100,000 +
1:100,000 +
1:100,000 -

Figure 5



Baseline Adjusted Flourescence 1/F1 (530nm)
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
-0.002
0
5
10
15
20
25
30
35
40
45
50
Cycle number
Mitoxanthrone control +
Mitoxanthrone control +
Mitoxanthrone control -
Mitoxanthrone control -
Dau 1:10 +
Dau 1:10 +
Dau 1:10 -
Dau 1:10 -
Dau 1:100 +
Dau 1:100 -
Dau 1:100 -
Dau 1:1,000 +
Dau 1:1,000 -
Dau 1:1,000 -
Dau 1:10,000 +
Dau 1:10,000 +
Dau 1:10,000 -
Dau 1:10,000 -
Dau 1:100,000 +
Dau 1:100,000 +
Dau 1:100,000 -
Dau 1:100,000 -